Prevalence and predictors for homo- and heterosubtypic antibodies against influenza a virus

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Abstract: Background: The effectiveness of trivalent influenza vaccination has been confirmed in several studies. To date, it is not known whether repeated exposure and vaccination to influenza promote production of cross-reactive anti-bodies. Furthermore, how strains encountered earlier in life imprint the immune response is currently poorly understood. Methods: To determine the prevalence for human homo- and heterosubtypic antibody responses, we scruti-nized serum samples from 305 healthy volunteers for hemagglutinin-binding and -neutralizing antibodies against several strains and subtypes of influenza A. Statistical analyses were then performed to establish the association of measured values with potential predictors. Results: It was found that vaccination not only promoted higher binding and neutralizing antibody titers to homosubtypic in fluenza isolates but also increased heterosubtypic human immune responses. Both binding and neutralizing antibody titers in relation with age of the donors mirrored the course of the different influenza strain circulation during the last century. Advanced age appeared to be of advantage for both binding and neutralizing titers to most subtypes. In contrast, the first virus subtype encountered was found to imprint to some degree subsequent antibody responses. Antibodies to recent strains, however, primarily seemed to be promoted by vaccination. Conclusions: We provide evidence that vaccinations stimulate both homo- and heterosubtypic immune responses in young and middle-aged as well as more senior individuals. Our analyses suggest that influenza vaccinations not only prevent infection against currently circulating strains but can also stimulate broader humoral immune responses that potentially attenuate infections with zoonotic or antigenically shifted strains.

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Prevalence and predictors for homo- and heterosubtypic antibodies against influenza A virus

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Running Title: Predictors of heterosubtypic antibodies

Keywords: Influenza virus, heterosubtypic antibodies, prevalence, predictors

Keypoints: Both binding and neutralizing heterosubtypic antibodies to non-human influenza A isolates can be found in most individuals. Their development is favored by age and frequent vaccination. Original antigenic sin was found to imprint but not to impair the homotypic antibody response.

Summary

Heterosubtypic antibodies to influenza A virus will be crucial for the development of a pan-influenza vaccine. Here we show that most individuals already possess heterosubtypic antibodies and that their generation is favored both by vaccination and age.

Abstract

Background: The effectiveness of trivalent influenza vaccination has been confirmed in several studies. To date, it is not known whether repeated exposure and vaccination to influenza promote production of cross-reactive antibodies. Further, how strains encountered earlier in life imprint the immune response is currently poorly understood.

Methods: To determine the prevalence for human homo- and heterosubtypic antibody responses, we scrutinized serum samples from 305 healthy volunteers for hemagglutinin-binding and -neutralizing antibodies against several strains and subtypes of influenza A. Statistical analyses were then performed to establish the association of measured values with potential predictors.

Results: It was found that vaccination not only promoted higher binding and neutralizing antibody titers to homosubtypic influenza isolates but also increased heterosubtypic human immune responses. Both binding and neutralizing antibody titers in relation with age of the donors mirrored the course of the different influenza strain circulation during the last century. Advanced age appeared to be of advantage for both binding and neutralizing titers to most subtypes. In contrast, the first virus subtype encountered was found to imprint to some degree subsequent antibody responses. Antibodies to recent strains, however, primarily seemed to be promoted by vaccination.

Conclusion: We provide evidence that vaccinations stimulate both homo and heterosubtypic immune responses not only in young and middle-aged, but also in more senior individuals. Our analyses suggest that influenza vaccinations not only prevent infection against currently circulating strains but can also stimulate broader humoral immune responses that potentially attenuate infections with zoonotic or antigenically shifted strains.

Background

The antibody response to influenza viruses is highly strain- and subtype-specific, and is primarily directed against the variable immunodominant apical epitopes on the hemagglutinin (HA) protein [1]. Vaccine-induced antibodies are therefore only effective against viruses closely related to the inoculated strains, and immunization needs to be revised annually in order to reflect the antigenic outfit of the viruses predicted to be predominant in the following influenza season. The breadth of the anti-influenza virus antibody response is further limited by the original antigenic sin (OAS) in that every immunization also boosts the memory response to the priming strain [2]. Indeed, Lessler and colleagues stated that in humans repeated exposure to different H3N2 strains increased antibody titers to strains encountered earlier in life [3].

Heterosubtypic antibodies, i.e. antibodies recognizing hemagglutinins from different subtypes, are rare [4]: in human pre-vaccination sera, only 0·01% of total serum IgG has been described to have heterosubtypic binding activity, one tenth of which being specific for the HA stem-epitope [4-6]. To establish the prevalence and predictors for heterosubtypic antibody responses, serum from 305 HIV-negative volunteers was collected in October 2009. In these sera we assessed both binding to five human and three non-human influenza isolates, and neutralizing antibody titers to five human and four non-human influenza isolates. These data were put into relation with epidemiological information acquired with a questionnaire at the blood draw.

Methods

Procedures

Sera from 305 randomly selected healthy volunteers were prospectively collected in Zurich, Switzerland, in late 2008 before the arrival of the H1N1 pandemic (H1pdm/09). The study was approved by the ethics committee of the University Hospital Zurich and written informed consent was obtained from all study participants. Self-reported demographic data including age, sex, travel history, the total number of estimated previous influenza vaccinations, estimated times of influenza infections (defined as 'flu' with more than three bedridden days and high fever), and potential contact to influenza-infected birds were collected on the day of the blood draft (an English translation of the questionnaire is given in the supplementary methods section). As antibodies binding to avian or extinct human HA subtypes are *bona-fide* cross-reactive, we determined binding antibody titers to recombinant trimeric HA protein from rH1pdm/09, rH2/57, rH4/56, rH5/04, rH7/79 and rH12/76 by ELISA and put them into relation to homotypic antibodies to rH1/34 and rH3/99. In addition, we assessed whether antibodies binding to recombinant protein can also bind immobilized purified H7N7 virions (H7vir/79, suppl. figure 3). The half-maximal effective dose (EC_{50}) was then determined by non-linear regression of optical density (OD) values, and used for statistical analyses as well as to establish correlations with the information collected in questionnaire.

Strains used for neutralization assays are described in the supplementary data. For evaluation, the reciprocal 50% inhibitory dose was determined by non-linear regression to the logarithm of serum concentration ($-\log(IC_{50})$), where possible. If most samples did not reach signal saturation, and constrained non-linear regression would have been prone for fitting artifacts, the inhibition percentage at the second serum dilution (1:90) was taken as a surrogate value. Statistical analyses were performed as outlined in the supplementary methods. R scripts used for this study are available at https://github.com/ozagordi/FluAbs.

Results

For the analysis of the prevalence and predictors of heterosubtypic antibodies, a random study population was compiled (n=305; table 1). At a serum dilution corresponding to the detection level of natural antibodies [7] and a relaxed threshold (threefold over background), between 99% and 100% of individuals, depending on the antigen tested, scored positive for serum antibodies binding, including all heterosubtypic HAs. Also at more stringent conditions of 1 in 90, 98-100% of individuals scored positive. If a more stringent threshold of a half-maximal signal was applied, heterosubtypic specificity could be monitored in 32-99% (at 1 in 30) and 0·3-79% (at 1 in 90) of the participants. The corresponding values are depicted in suppl. figure 1.

With the exception of rH12/76, binding titers to the avian strains (rH4/56, rH5/04 and rH7/79) were low compared to human HAs. Low titers were also observed against rH1pdm/09 that had not yet arrived in Switzerland when the blood was collected. Titers against human rH2/57 that became extinct in 1968 were also found to be relatively low, although distributed over a wide range of values.

To determine the direct antiviral activity of these antibodies we performed *in vitro* neutralization assays against H1/34, H1/07, H2/57, H3/07, H3/68, H4/56, H5/04 and H7/79 viruses. The highest neutralizing titers were found against homologous human strains (H1/34, H1/07, H2/57, H3/68 and H3/07; suppl. figure 1C and table S1). Titers to avian H4/56 were much lower and the barely detectable neutralizing activity to H5 and H7 even prevented computation of the logIC_{50} values. Instead, the percentage of inhibition at the first dilution (1 in 90) was taken as comparative proxy value for these viruses (suppl. figure 1D).

ELISA titers and neutralizing activity correlated well in that high titers in ELISA are indicative for high neutralizing activity (table S2). Vaccinated individuals clearly displayed higher binding and neutralizing titers against human subtypes H1/34, while still showing increased titers against rH2/57, rH3/99 and H3/68 (figure 1 and 2), albeit less markedly. As highlighted in the regression analysis, some of these differences may be explained by age alone (table 2). Vaccinated individuals also displayed significantly higher neutralizing titers

against H1/07 and H3/07 than non-vaccinated individuals. They also displayed higher binding antibody titers to heterosubtypic rH1pdm/09 and rH5/04 (figure 1). Vaccination showed no or weaker effect on binding titers against the remaining three tested heterosubtypic rHAs.

The effect of age, influenza episodes and gender was also analyzed (table 3 and 4). As an exploratory data analysis, we stratified the age in three different categories according to the different influenza pandemics [baseline: born 1969-2009 (age 0-40), category 1: 1958-1968 (age 41-51), category 2: 1919-1957 (age 52-90)]. After adjusting the model for age, influenza episodes and gender, vaccination still remained a predictor for higher binding and neutralizing titers to the same subtypes as in the two-sample t test. However, the impact of vaccination was lost for rH2/57 binding and H3/68 neutralizing titers in the adjusted models (table 3 and 4).

We then performed linear regression to correlate the age of donors and the vaccination status with binding and neutralizing antibody titers (suppl. figures 2 and table 2).

Binding titers to human strains all increased with age, while increasing only in two out of five non-human strains, all of these phylogenetically close to the human ones (H1pdm/09 and H5/04). Neutralizing titers were also found to increase with age in two out of five human strains tested (H2/57 and H3/68). On the other hand, titers to heterosubtypic strains did not display a dependence on age.

Similarly, vaccination showed a positive correlation in binding and neutralizing titers to most human strains, with the exception of the neutralizing titers to H3/68, which were independent of vaccination (figure 3, table 2). Titers to H2/57 were distinct, as binding titers did not depend on vaccination, and neutralizing titers showed a modest interaction between age and vaccination status. Specifically, neutralizing titers to H2/57 increased with age for both vaccinated and non-vaccinated groups, but were more pronounced in the vaccinated group. As both H2 and H5 subtypes have never been included in the vaccines, the fact that vaccination had a positive correlation with neutralizing titers to these subtypes can be

explained by their phylogenetic proximity to H1 subtype, which is part of the widely used split vaccine formulation.

In order to expose differences between age groups that would go undetected in linear regression, we performed loess smoothing on antibody titers in relation to age separately for vaccinated and non-vaccinated donors. Neutralizing titers to recent human isolates (H1/07 and H3/07) were higher in vaccinees, but overall not influenced by age (as already suggested by linear regression). However, non-linear regression revealed that the youngest participating donors (approximately below age 30) clearly showed the highest titers (figure 3). Similar findings were made for binding titers to recently isolated H3/99. This was in contrast to the behavior observed against older isolates (H2/57 and H3/68); here the best neutralizing titers were found in those donors who were in their first decade of life when the corresponding isolate has been circulating (age 50 to 61 and age 41 to 50, respectively). The same trend emerged for binding titers to H2/57. On the other hand, for an older H1 isolate (H1/34) this effect was less pronounced for both neutralizing and binding titers.

Discussion

Our study demonstrates that heterosubtypic antibodies against influenza viruses, i. e. antibodies recognizing hemagglutinin from multiple subtypes, can be found in most exposed or immunized individuals. These findings corroborate previous findings made by biochemical analysis of a limited number of samples [4, 8]. We have found that vaccination not only enhanced antibody responses to subtypes that are currently circulating, and are therefore included in the vaccine, but also augmented binding and neutralizing antibody titers to heterosubtypic subtypes. Senior people had higher antibody titers to old homosubtypic influenza isolates than their younger counterparts, whose antibody response appeared to favor more recent isolates. Age was also found to be a predictor with a positive coefficient for higher antibody titers to heterosubtypic isolates. In contrast, the number of self-reported influenza episodes did not correlate with higher antibody titers, which, given

the unreliability of the parameter, is not surprising. Moreover, since vaccinations, like influenza episodes, also accumulate with age and may not always be reported accurately, it is hard to disentangle their effect on the antibody titers. For this reason, vaccination status was reported as binary (vaccinated/non vaccinated), discarding differences in the number of vaccinations.

As the participants of our study have not recently been vaccinated, their antibody repertoire is not skewed towards a recently inoculated vaccine-strain. Attention is therefore focused on the serum antibody composition present when infection is most likely to occur. The use of viable viruses for neutralization assays rather than highly-neutralization sensitive pseudotyped virions further assured that the neutralizing activity detected is biologically relevant [9].

At the time of serum sampling, H1pdm/09 had not yet reached Switzerland. Potential H1pdm/09-related induction of broadly cross-reactive antibodies [10] is therefore unlikely to have skewed our results. Yet, our data is consistent with findings that H1pdm/09 possesses an epitope that is shared with the H1N1 virus strains circulating before 1957: individuals born before 1957 had higher titers to H1pdm/09 than younger participants [11, 12]. Although the binding titers were expected to increase with age in the non-vaccinated cohort, no peak in elderly appeared in non-parametric smoothing. Both observations, however, could be explained by the low sample size in this age group (figure 3).

Since we used a novel neutralization assay we cannot give predictions for protection. In the hemagglutinin inhibition assays (HIA), a serum dilution of 1 in 40 is considered protective while comparable values were found for an ELISA-based and a colorimetric microneutralization assay (MN) but these values are not applicable to our assay [13] in particular since it was also shown that ELISA values and HIA values do not correlate, not even within the same subtypes [14]. Moreover, since the majority of heterosubtypic antibodies was found to bind a conserved epitope in the stem of the HA protein and did not interfere with hemagglutination [15, 16], they would not be detected by HIA. Consequently,

no direct predictions for heterosubtypic protection can be drawn from our dataset at this point. Further research will be required to define this relationship.

In contrast, the large number of serum samples tested for both binding and neutralization allowed us the establishment of a linear relationship: increasing titers of binding antibodies correlate statistically significant with increasing neutralizing titers (table S2). The correlation coefficient R for extinct human strains was reasonable high especially in rH2/57 vs. H2/57 (R=0·43), whereas isolates with very distant isolation years, H3/68 vs. rH3/99 did not correlate at all, despite belonging to the same subtype.

Lessler and colleagues found that repeated exposure to different H3N2 strains increased antibody titers to those strains encountered earlier in life while progressively fewer specific antibodies to subsequent infection are made with age [3]. We could confirm this finding in that the neutralizing and binding titers were the highest in those individuals who were in their first decade of life during the period of time when the corresponding subtype was circulating. Older individuals also had significantly more binding and neutralizing antibodies against old than more recent isolates (e.g. H3/99; suppl. figure 2), which may reflect original antigenic sin [17]. In contrast, in line with other studies [18] we found antibody titers to the last pre-study isolates (’07) to be the same in all age groups.

In elderly individuals, vaccination increased the probability of having heterosubtypic antibodies. Thus, while senescence of the immune system presumably contributed to a poorer response to recent isolates, age was advantageous for the development of heterosubtypic antibodies (to both human and non-human isolates). Vaccination always improved titers in the oldest or middle age group compared to young individuals. Although the age at which the vaccinations were received was not assessed, most individuals received them in the previous 5-10 years. In fact, influenza vaccination was very uncommon in Switzerland prior to the late nineties. [19]. Our findings show that vaccination is of value also in more seasoned individuals, and that its benefit is not just the result of vaccinations at younger age. This provides additional, albeit indirect, support for annual vaccinations in elderly.

Although most humans have low levels of heterosubtypic antibodies, they are still susceptible to infection with antigenically drifted or shifted influenza A strains, indicating that these antibodies probably are not protective. Yet such antibodies are likely to attenuate disease but, to prove this hypothesis, large clinical trials would need to be conducted. The findings of our study nonetheless clearly indicate that novel vaccination strategies targeting the conserved epitopes of influenza hemagglutinin could profit from pre-existing antigen-experienced heterosubtypic B cells. Our data also unambiguously support yearly vaccination as neutralizing antibody titers increase over time in elderly and younger people and furthermore, more heterosubtypic antibodies are induced.

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Figure legends

Figure 1. Vaccination-dependent differences in -log(EC_{50}) titers against recombinant human and avian influenza hemagglutinin. Serum antibody reactivities to the indicated immobilized recombinant hemagglutinin were assessed by ELISA. Two-sample t test analyses of the data were performed and showed higher binding antibody titers to the human subtypes rH1/34 and rH3/99, to heterosubtypic rH1pdm/09, rH2/57 and to avian heterosubtypic rH5/04 titers in the vaccinated cohort. Values are shown as logarithm of the reciprocal serum dilution giving a half-maximal signal (-logEC_{50}). Median and p-values of each group are indicated by numbers. Boxplots represent median and IQR, whiskers depict lower or upper quartile ± 1·5 x IQR.

Figure 2. Differences in serum half-maximal inhibitory titer -log(IC_{50}) in vaccinated and non-vaccinated individuals against human and avian influenza subtypes. Serum antibody neutralizations against the indicated viral subtypes were assessed in neutralization assays. Two sample t-test analysis were performed and showed higher neutralizing antibody titers to all homotypic viruses (H1/07, H1/34, H3/68 and H3/07) and to heterosubtypic avian virus H5/04 in the vaccinated cohort. No improvement in titers for human heterosubtypic H2/57 and for avian H4/56 or H7/79 viruses was found. Neutralizing data from figure 1 was used for this analysis. Values are shown as logarithm of the reciprocal serum dilution giving a half-maximal inhibitory concentration, and as inhibition percentage against H5/57 and H7/79 at a serum dilution of 1 in 90. Median and p-values are indicated. Boxplots indicate median and IQR. Whiskers include lower or upper quartile ± 1·5 x IQR.

Figure 3: Loess smoothing of antibody titers. (A) The binding antibody titers for rH3/99, but not for rH1/34 or rH2/57, were lower in more seasoned individuals. Smoothing discovers steep increase of -log(EC_{50}) around age 40 for rH2/57. For rH1/34, smoothing displayed results similar to linear regression. Highest -log(EC_{50}) titers to rH3/99 are depicted in the age group of the 21-27 years old individuals. Values are shown as logarithm of the reciprocal serum dilution in correlation with age. **(B)** Neutralizing titers to H1/34

(vaccinated), H2/57 (vaccinated and non-vaccinated) and H3/68 (vaccinated and non-vaccinated) increased with increasing age. -log(IC_{50}) to H1/07 (vaccinated) decreased with increasing age (cf. suppl. figure 2). Remaining subtypes do not show slopes different from zero. Smoothing of -log(IC_{50}) for H2/57 discovered a peak at age 50. Values are shown as logarithm of the reciprocal serum dilution giving a half-maximal inhibitory concentration in correlation with age, and, separated by bold frame lines, as inhibition percentage against H5/57 and H7/79 at a serum dilution of 1 in 90. Blue triangles and lines represent the vaccinated, black squares and lines the non-vaccinated cohort. Grey shaded areas indicate the confidence band. Significance codes (testing the slope being different from zero): *** p<0·001, ** p<0·01, * p<0·05.

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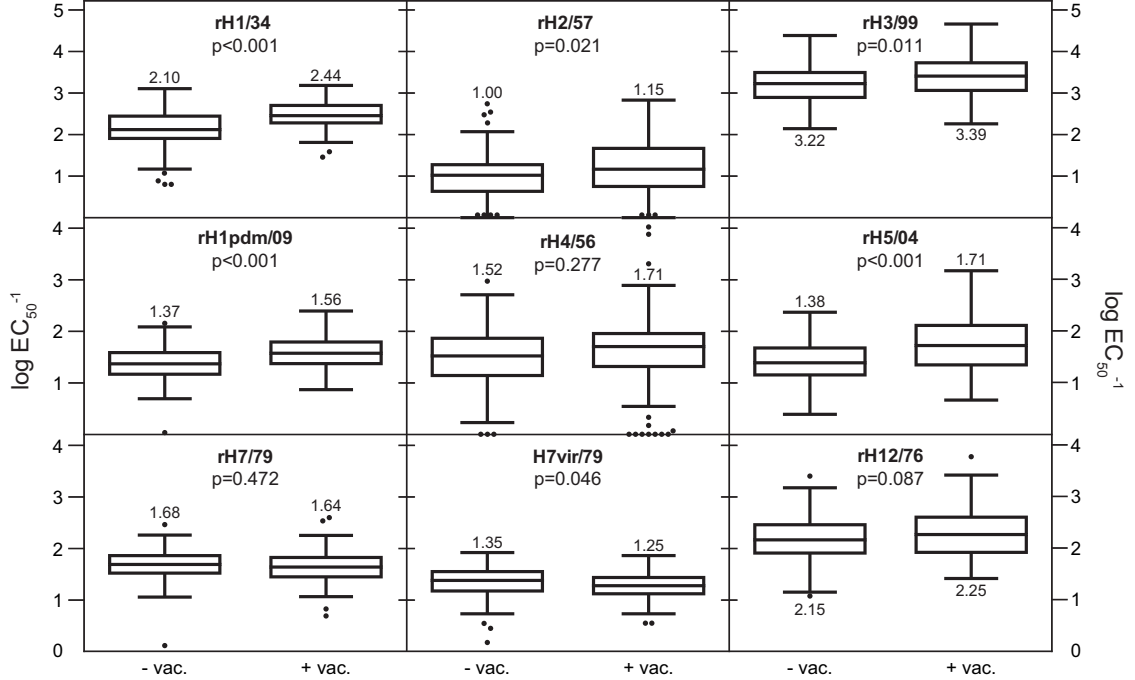
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2.44
rH2/57
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1.15
rH3/99
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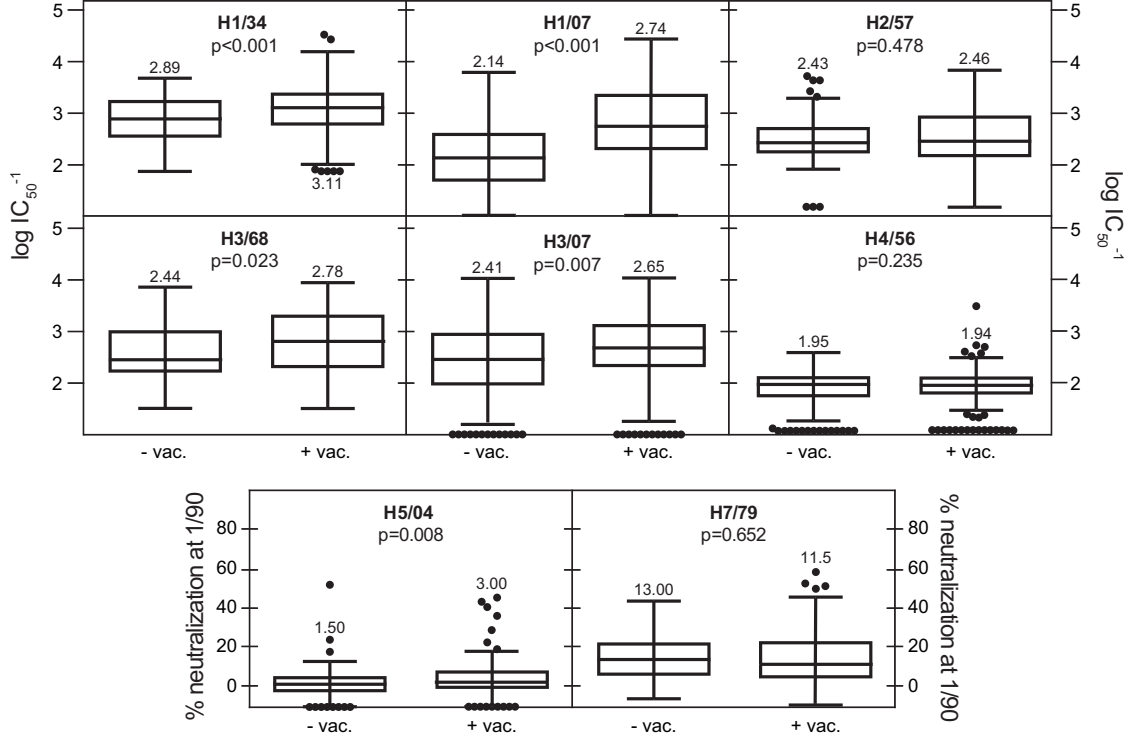
3.22
3.39
rH1pdm/09
p<0.001
1.37
1.56
rH4/56
p=0.277
1.52
1.71
rH5/04
p<0.001
1.38
1.71
rH7/79
p=0.472
1.68
1.64
H7vir/79
p=0.046
1.35
1.25
rH12/76
p=0.087
2.15
2.25
log EC50-1
log EC50-1
- vac.
+ vac.
- vac.
+ vac.
- vac.
+ vac.


H1/34
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2.89
3.11
H1/07
p<0.001
2.14
2.74
H2/57
p=0.478
2.43
2.46
H3/68
p=0.023
2.44
2.78
H3/07
p=0.007
2.41
2.65
H4/56
p=0.235
1.95
1.94
log IC50-1
- vac.
+ vac.
H5/04
p=0.008
1.50
3.00
H7/79
p=0.652
13.00
11.5
% neutralization at 1/90

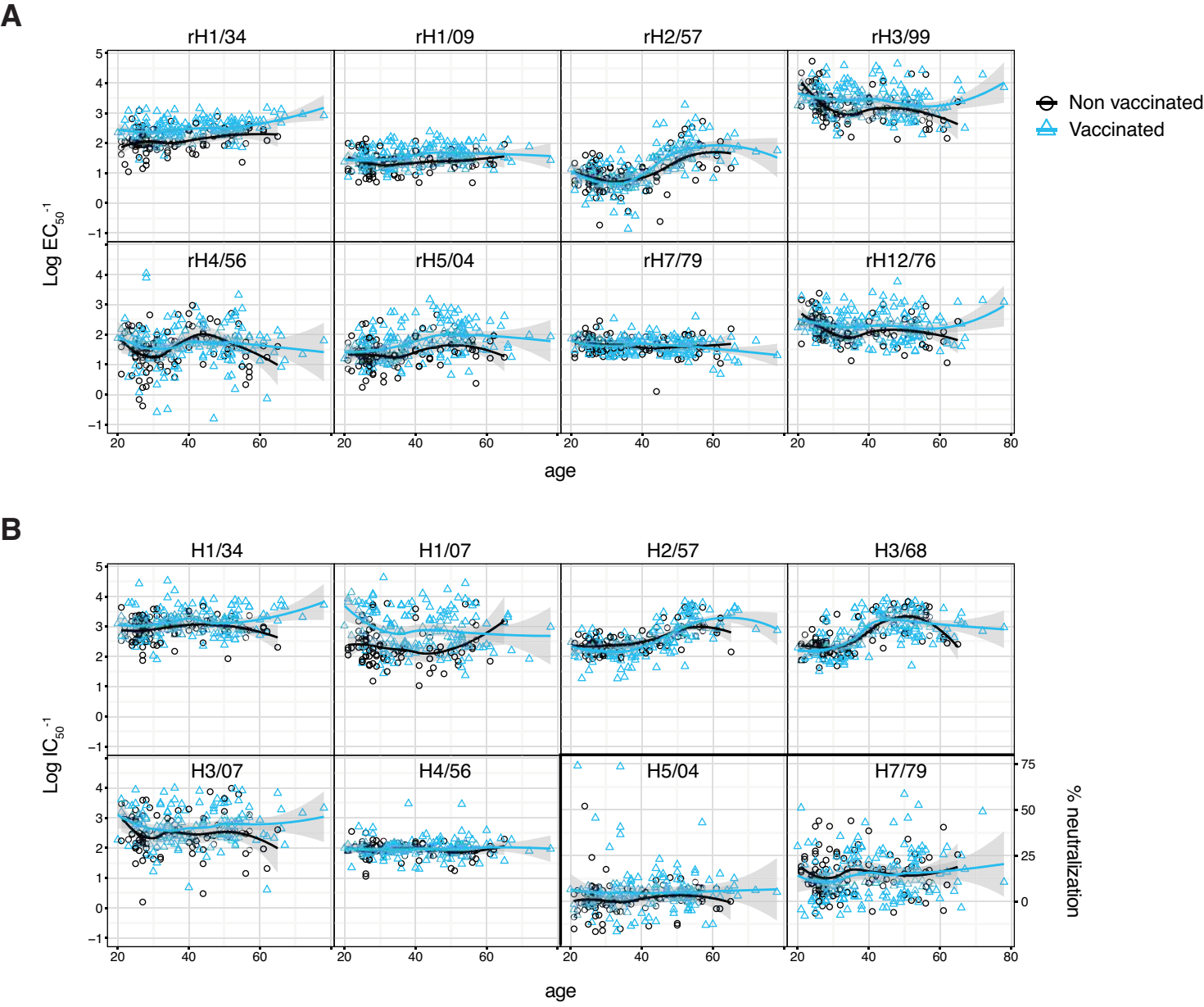

A
rH1/34
rH1/09
rH2/57
rH3/99
rH4/56
rH5/04
rH7/79
rH12/76
Log EC50 -1
age
Non vaccinated
Vaccinated
B
H1/34
H1/07
H2/57
H3/68
H3/07
H4/56
H5/04
H7/79
Log IC50 -1
% neutralization
age

Table 1. Cohort characteristics

Total study participants included in the study (n)	305
Gender (female)	156 (51·15%)
Median age [interquartile range (IQR)]	36 [28-49]
Median vaccinations [IQR]	2 [0-5]
Median episodes [IQR]	2 [0-3]
Contact to FPV-infected poultry	3 (0·98%)
Travel to rural region in southeastern Asia	23 (7·54%)
Novartis vaccine trial	17 (excluded)
Swine flu (H1pdm/09) infection	0

Table 2. Linear regression analyzing the impact of age and vaccination on binding and neutralizing antibody titers.

Linear regression analyzing the impact of age and vaccination on binding titers -log(EC_{50}) and neutralizing titers -log(IC_{50})

Subtype		rHAs and strains	Intercept	Age (α)	Vaccination (β)	Interaction (γ)
Human	Binding	rH1/34	1·80 ± 0·067	0·0081 ± 0·0017	0·33 ± 0·041	
		rH2/57		0·029 ± 0·0007		
		rH3/99	3·58 ± 0·099	-0·0096 ± 0·0025	0·21 ± 0·062	
	Neutralization	H1/34	2·92 ± 0·043		0·21 ± 0·054	
		H1/07	2·34 ± 0·070		0·54 ± 0·087	
		H2/57	1·83 ± 0·12	0·019 ± 0·0032	-0·55 ± 0·16	0·012 ± 0·0040
		H3/68	1·64 ± 0·097	0·027 ± 0·0024		
		H3/07	2·47 ± 0·065		0·24 ± 0·082	
Heterosubtypes	Binding	rH1pdm/09	1·19 ± 0·065	0·0047 ± 0·0016	0·18 ± 0·041	
		rH4/56	1·62 ± 0·68			
		rH5/04	0·93 ± 0·099	0·013 ± 0·0025	0·27 ± 0·062	
		rH7/79	1·64 ± 0·36			
		rH12/76	2·22 ± 0·62			
	Neutralization	H5/04	1·36 ± 1·05		3·62 ± 1·34	
		H7/79	11·5 ± 17·5			

rHAs, recombinant hemagglutinin.

Binding titers -log(EC_{50}) and neutralizing titers -log(IC_{50}) were modeled as a linear function of age, vaccination and their interaction (intercept + $\alpha \times$ age + $\beta \times$ vaccination + $\gamma \times$ age $\times$ vaccination). The age is given in years and vaccination is a binary dummy variable. Starting from the complete model, coefficients not significantly differ from zero (at the 1% level) were dropped and the model estimated again. The table reports the parameter estimates for the final model. Where no effect of age and/or vaccination was detected, median and interquartile range are reported in the intercept column.

Table 3. Analysis of binding titers with respect to age group and vaccination status.

Table 3. Multivariable regressions analyzing binding titers -log(EC_{50}) in relation with vaccination and age.

rHA	Characteristics	Regression Coefficient (95% CI)	p-value
rH1/34	Vaccination (≥1)	0·35 (0·27 to 0·43)	<0·001
	Age (41-51)	0·11 (0·016 to 0·21)	0·023
	Age (52-90)	0·23 (0·13 to 0·33)	<0·001
rH2/57	Vaccination (≥1)	0·12 (-0·10 to 0·33)	0·267
	Age (41-51)	0·53 (0·28 to 0·78)	<0·001
	Age (52-90)	1·12 (0·86 to 1·38)	<0·001
rH3/99	Vaccination (≥1)	0·19 (0·070 to 0·31)	0·002
	Age (41-51)	-0·11 (-0·26 to 0·038)	0·145
	Age (52-90)	-0·25 (-0·40 to -0·095)	0·002
rH1pdm/09	Vaccination (≥1)	0·20 (0·11 to 0·29)	<0·001
	Age (41-51)	0·15 (0·048 to 0·26)	0·004
	Age (52-90)	0·14 (0·033 to 0·25)	0·011
rH4/56	Vaccination (≥1)	0·062 (-0·14 to 0·26)	0·543
	Age (41-51)	0·34 (0·096 to 0·58)	0·006
	Age (52-90)	0·20 (-0·055 to 0·45)	0·125
rH5/04	Vaccination (≥1)	0·28 (0·16 to 0·40)	<0·001
	Age (41-51)	0·35 (0·21 to 0·49)	<0·001
	Age (52-90)	0·33 (0·18 to 0·48)	<0·001
rH7/79	Vaccination (≥1)	-0·015 (-0·086 to 0·055)	0·667
	Age (41-51)	-0·041 (-0·13 to 0·044)	0·347
	Age (52-90)	-0·065 (-0·15 to 0·020)	0·148
rH12/76	Vaccination (≥1)	0·11 (-0·0037 to 0·23)	0·058
	Age (41-51)	-0·027 (-0·16 to 0·11)	0·704
	Age (52-90)	-0·11 (-0·25 to 0·034)	0·052

Reference age: 0-40; rHAs, recombinant hemagglutinin.

Table 4. Analysis of neutralizing titers with respect to age group and vaccination status.

Table 4. Multivariable regressions analyzing neutralizing titers -log(IC_{50}) in relation with vaccination and age

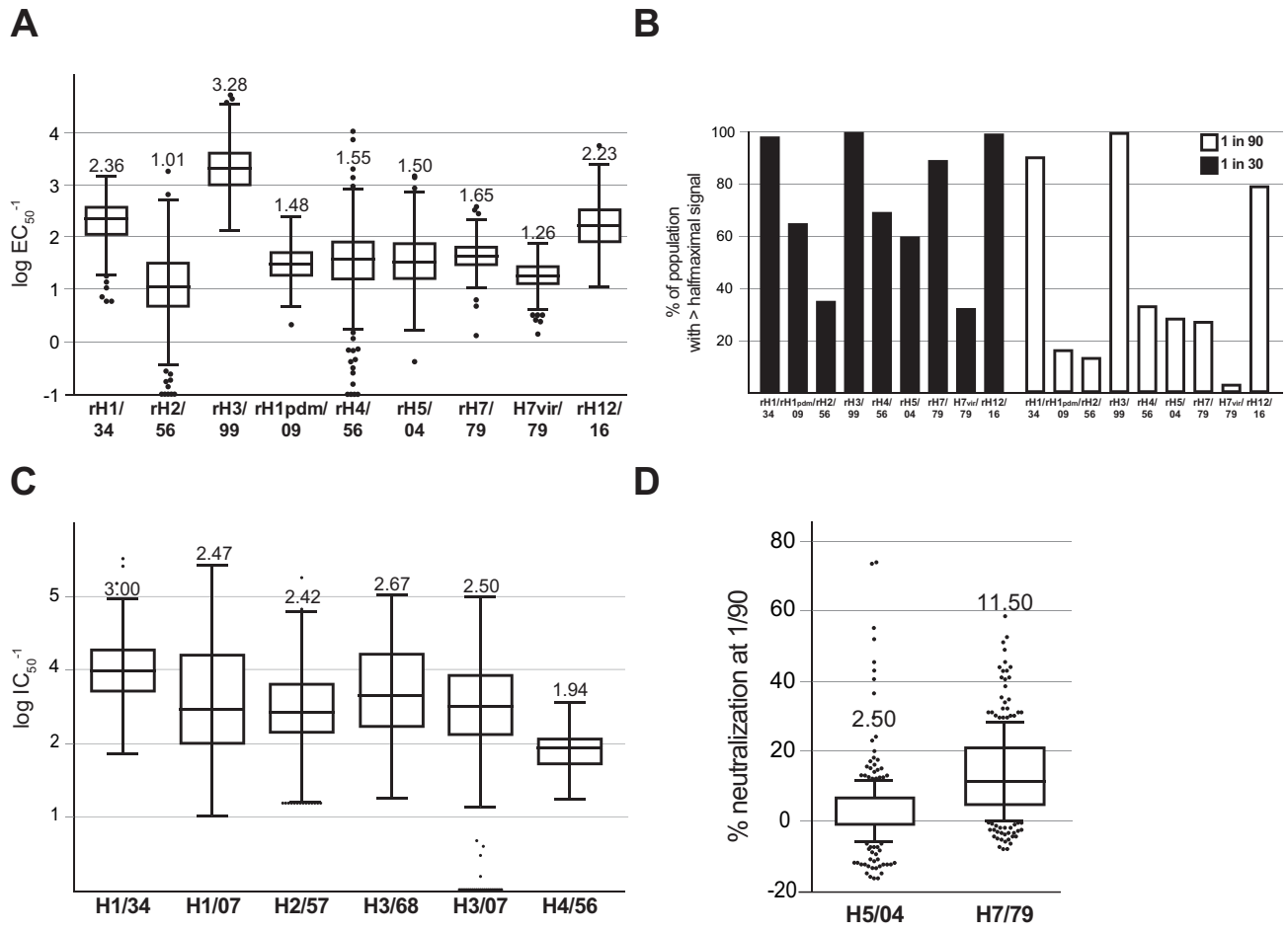
Virus	Characteristics	Regression Coefficient (95% CI)	p-value
H1/34	Vaccination (≥1)	0·27 (0·15 to 0·39)	<0·001
	Age (41-51)	0·017 (-0·12 to 0·16)	0·809
	Age (52-90)	0·099 (-0·048 to 0·25)	0·185
H1/07	Vaccination (≥1)	0·66 (0·47 to 0·85)	<0·001
	Age (41-51)	-0·073 (-0·30 to 0·15)	0·524
	Age (52-90)	-0·074 (-0·31 to 0·16)	0·536
H2/57	Vaccination (≥1)	-0·085 (-0·18 to 0·013)	0·089
	Age (41-51)	0·46 (0·34 to 0·57)	<0·001
	Age (52-90)	0·96 (0·83 to 1·082)	<0·001
H3/68	Vaccination (≥1)	0·022 (-0·094 to 0·14)	0·708
	Age (41-51)	0·85 (0·71 to 0·99)	<0·001
	Age (52-90)	0·73 (0·59 to 0·88)	<0·001
H3/07	Vaccination (≥1)	0·40 (0·106 to 0·69)	0·008
	Age (41-51)	0·094 (-0·26 to 0·45)	0·597
	Age (52-90)	-0·096 (-0·46 to 0·27)	0·604
H4/56	Vaccination (≥1)	0·034 (-.058 to .13)	0·464
	Age (41-51)	0·085 (-.026 to .20)	0·133
	Age (52-90)	0·14 (.021 to .25)	0·021
H5/04	Vaccination (≥1)	3·43 (0·76 to 6·10)	0·012
	Age (41-51)	0·71 (-2·50 to 3·92)	0·664
	Age (52-90)	0·39 (-2·94 to 3·72)	0·818
H7/79	Vaccination (≥1)	-1.059 (-4·091 to 1·97)	0·492
	Age (41-51)	0·71 (-2·95 to 4·34)	0·708
	Age (52-90)	3·32 (-0·64 to 7·11)	0·085

Reference age: 0-40.

Supplementary Figures

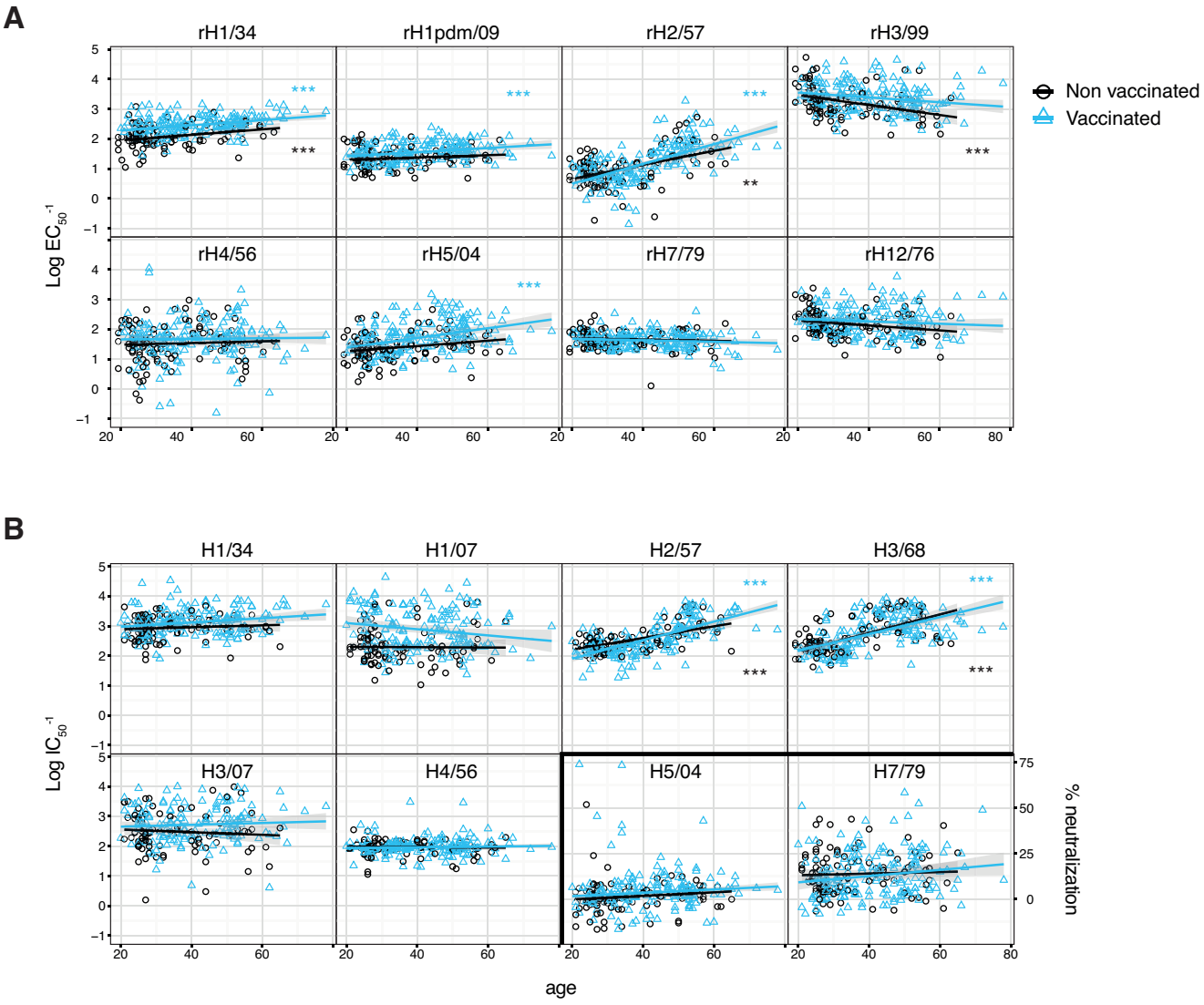
Suppl. Figure 1:

Figure 1. Prevalence and distribution of homo- and heterosubtypic serum antibodies.



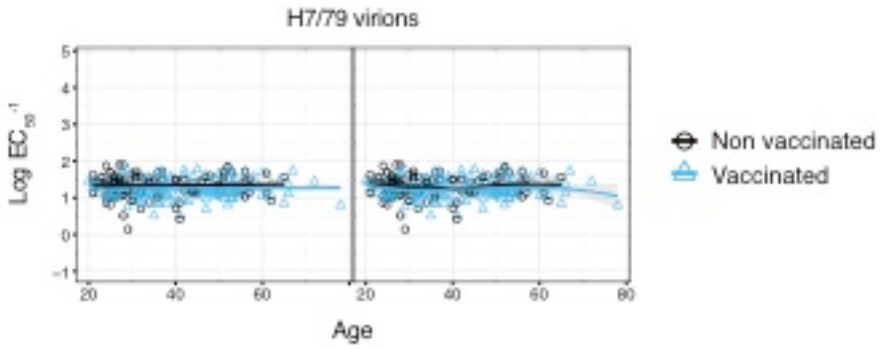
(A) Serum antibody reactivities against the indicated immobilized recombinant hemagglutinin were assessed by ELISA and displayed as the logarithm of the reciprocal serum dilution giving a half-maximal effective dose -log(EC_{50}). (B) Percentage of participants displaying ELISA signals greater than half-maximal value against the indicated HA subtype at a serum dilution of either 1 in 30 (bold-faced bars) and 1 in 90 (open-faced bars). (C) Neutralizing serum antibody titer to human and avian virus isolates plotted as the logarithm of the reciprocal serum dilution giving half-maximal inhibition (-logIC_{50}). (D) Percent neutralization of H5/57 and H7/79 viruses at a serum dilution of 1 in 90 for viruses whose IC50 could not be estimated. Neutralization was tested at a multiplicity of infection of 2-6. Boxplots in (A), (C) and (D) indicate median and interquartile ranges (IQR), whiskers include lower or upper quartile ± 1·5 x IQR.

Suppl. Figure 2: Linear regression of binding and neutralizing antibody titers plotted against age in vaccinated and non-vaccinated donors.



(A) Binding titers. The -log(EC_{50}) values for rH3/99, but not for rH1/34 or rH2/57, were lower in more seasoned individuals. Smoothing discovers steep increase of -log(EC_{50}) around age 40 for rH2/57. Highest -log(EC_{50}) titers to rH3/99 are depicted in the age group of the 21-27 years old individuals. Values are shown as logarithm of the reciprocal serum dilution in correlation with age. **(B) Neutralizing titers.** Neutralizing titers to H1/34 (vaccinated), H2/57 (vaccinated and non-vaccinated) and H3/68 (vaccinated and non-vaccinated) increased with increasing age. -log(IC_{50}) to H1/07 (vaccinated) decreased with increasing age. Remaining subtypes do not show slopes different from zero. Values are shown as logarithm of the reciprocal serum dilution giving a half-maximal inhibitory concentration, and as inhibition percentage against H5/57 and H7/79 at a serum dilution of 1 in 90. Blue triangles and lines represent the vaccinated, black squares and lines the non-vaccinated cohort. Grey shaded areas indicate the confidence band. Significance codes (testing the slope being different from zero): *** p<0·001, ** p<0·01, * p<0·05.

Suppl. Figure 3: Linear regression and smoothing of binding antibody titers to virions of H7/79 directly coated to ELISA plates



Left panels: linear regressions; right panels: loess smoothing.

Supplementary Tables

Supplementary Table 1. Prevalence and distribution of homo- and heterosubtypic serum antibodies.

S1. Prevalence and distribution of homo- and heterosubtypic serum antibodies.

rHAs	-log(EC_{50})Median [IQR]
rH1/34	2·36 [2·06 to 2·57]
rH1/pdm09	1·48 [1·27 to 1·73]
rH2/57	1·06 [0·69 to 1·51]
rH3/99	3·28 [2·98 to 3·64]
rH4/56	1·55 [1·18 to 1·91]
rH5/04	1·50 [1·21 to 1·88]
rH7/79	1·65 [1·48 to 1·83]
rH12/76	2·23 [1·91 to 2·51]

HAs	-log(IC_{50})Median [IQR]
H1/34	3·00 [2·71 to 3·28]
H1/07	2·47 [2·015 to 3·21]
H2/57	2·42 [2·16 to 2·81]
H3/68	2·67 [2·23 to 3·21]
H3/07	2·50 [2·13 to 2·94]
H4/56	1·94 [1·74 to 2·07]
H5/04 *	2·50 [-1·00 to 6·50]
H7/79 *	11·50 [4·50 to 21·00]

rHAs, recombinant hemagglutinin.
* [% neutralization at 1/90]

Supplementary Table 2. Linear regressions analyzing relation of binding vs. neutralizing titers.

S2. Linear regressions analyzing relation of binding titers -log(EC_{50}) vs. neutralizing titers -log(IC_{50})

Strains vs. rHAs	Regression Coefficient β (95% CI)	p-value
H1/07 vs. rH1/pdm09	0·12 (0·068 to 0·16)	<0·001
H1/34 vs. rH1/34	0·29 (0·20 to 0·38)	<0·001
H1/34 vs. rH1/pdm09	0·15 (0·069 to 0·24)	<0·001
H1/07 vs. rH1/34	0·17 (0·11 to 0·22)	<0·001
H2/57 vs. rH2/57	0·69 (0·53 to 0·85)	<0·001
H3/68 vs. rH3/99	-0·062 (-0·15 to 0·026)	0·168
H3/07 vs. rH3/99	0·14 (0·095 to 0·18)	<0·001
H4/56 vs. rH4/56	0·52 (0·29 to 0·74)	0·011
H5/04 vs. rH5/04	0·0074 (0·0017 to 0·013)	<0·05
H7/79 vs. rH7/79	0·000088 (-0·0025 to 0·0027)	0·948
H7/79 vs. H7vir/79	0·0024 (-0·00018 to 0·0050)	0·068

Reported are the results of linear regression of binding vs. neutralizing titers. The p-value is reported for the null hypothesis: $\beta = 0$.

Supplementary methods

Study design and participants

The ethics committee of the University Hospital Zurich approved the study (ref. no. EK-17-42) and written informed consent was obtained from all study participants. Enrollment ran for the most part from October 20, 2009 to October 30, 2009 (before the arrival of the swine-flu pandemic virus H1pdm/09 in Switzerland). A minor portion (n=13) of blood samples was collected one year before. All samples were obtained before the start of the seasonal vaccine campaign 17 (5·28%) donors who were enrolled in the 2009 Novartis clinical trial for the H1/pdm09 vaccine, were excluded from our analysis [1].
This included age, categories of the number of approximate previous vaccinations, influenza exposure history (including potential contact to fowl plague infected poultry), enrollment in the 2009 Novartis vaccine trial for the H1pdm/09 vaccine and recent travel to rural regions in southeastern Asia. The number of influenza infections was subjectively apprised as influenza-like disease lasting for more than five days, and is not supported by laboratory data.

Translation of the questionnaire filled out at the time of the blood drawing:

1. How often in your life do you think you have had an influenza infection? With influenza infection we refer to all 'flus' that lasted longer than 4 days, that were accompanied by severe malaise and high fever, and that left you bedbound for more than 3-4 days.
2. How often have you been vaccinated against influenza?
3. Have you already been vaccinated against influenza this year?
4. Have you been vaccinated against the swine flu?
5. Have you been diagnosed with swine flu and did a laboratory confirm this diagnosis?
6. Have you been infected by swine flu?
7. Did you stay longer than 3 weeks in rural areas of Southeast Asia?
8. Have you knowingly been in contact with influenza infected fowl or poultry? Avian influenza is also referred to as 'fowl plague'.

Statistical analyses

STATA 12 SE (StataCorp, College Station, TX) and R (2.15.2) software were used for association and correlations studies. GraphPad Prism 6 and Adobe Illustrator CS 5.1 were used for generation of blots.
Vaccination analysis (figure 2 and 3) was performed using two-sample t tests, participant numbers were as follows: 0 vaccinations (n=104), ≥1 vaccination (n=169) and missing declaration (n=32). Influenza episodes analyses were studied using simple and linear regression; 0 episodes (n=33), ≥1 episodes (68), ≥1 episodes and vaccinations (n=169) these were excluded from the episode study and missing declaration (n=35) data not shown.
Age categories were binned into: 0-40 y (n=184), 41-51 y (n=65) and 52-90 (n=56).

Multivariable analyses were performed using multiple linear regressions.

Robust linear regressions in figures 4-7 were computed with rlm (MASS package), smoothing with loess (stats package) and displayed with ggplot2. Smoothing is noisier in proximity of the endpoints. Since the smoothing is done on less data, few points can influence the estimate heavily. For this reason the confidence regions grow at the borders, and one must be cautious in not over interpreting this behavior.

Regression analysis of the full model was performed with glm (stats package).

In a first analysis, linear regression was performed separately on vaccinated and non-vaccinated donors with the model: binding titer = intercept + $\alpha \times$ age. By estimating the age effect separately, potential distortions induced by repetitive trivalent inactivated influenza vaccinations (TIV) could be assessed.
Including the vaccination status as a covariate allowed us to simultaneously estimate the effect of age and vaccination and a potential interaction between them. To this end, we started estimating the coefficients of the full model: binding titer = intercept + $\alpha \times$ age + $\beta \times$ vaccination + $\gamma \times$ age $\times$ vaccination, where the age is given in years and vaccination is a binary dummy variable.
In order to avoid over fitting, the complexity of the model was iteratively reduced by removing the coefficients not significantly different from zero and re-estimating the others. We required the p-value for the regression coefficients to be lower than 1% in order to keep them in the model. This informal calibration of the significance threshold also constitutes an attempt to address the multiple testing issue [2].
Scripts used for this study are available at https://github.com/ozagordi/FluAbs

Blood samples

Full blood was allowed to coagulate at RT for at least 30 min before serum was collected by centrifugation (at 20 °C at 2'500 x g for 15 min), and stored in 1mL aliquots at -80 °C. Before use, aliquots of sera were thawed and heat inactivated at 56 °C for 30 min and supplemented with 0.1% NaN_3 as preservative. Heat inactivated serum was stored at 4 °C.

Cells

Madin Darby canine kidney (MDCK) cells were purchased from the American Type Culture Collection (ATCC®: CCL-34™) and used for all cell-based assays. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with heat inactivated 10% fetal bovine serum, and 50 U/mL penicillin and 50 µg/mL streptomycin.

Virus preparation and concentration

Influenza viruses were propagated either on MDCK or in d11 embryonated hen eggs, and harvested 24 to 48 h (MDCK cells) or 48 h (eggs) post infection. For ELISA using virions from A/FPV/Bratislava/79(H7N7) (H7vir/79), virus was collected from cell supernatant by ultracentrifugation through a 32% sucrose cushion (2 h at 4 °C at 28'000 rpm; swing out rotor SW28).

Generation of recombinant HA

Recombinant HAs (rHAs) were obtained from following strains: A/Puerto Rico/8/34(H1N1) (rH1/34), A/California/04/09(H1N1) (rH1pdm/09), A/Japan/305/57(H2N2) (rH2/57), A/Moscow/10/99(H3N2)(rH3/99), A/Duck/Czechoslovakia/56(H4N6) (rH4/56), A/Vietnam/1203/04(H5N1) (rH5/04), A/FPV/Bratislava/79(H7N7) (rH7/79) and A/Duck/Alberta/35/76 (H12N5) (rH12/76).
The HA-constructs were either generated from viral RNA according to Hoffman or *de novo* synthesis according to the published sequence or by already cloned cDNAs received from other labs [3]. In brief, viral RNA was isolated from cell supernatant using RNeasy Mini Kit columns (Qiagen). Following reverse transcription using Superscript II reverse transcriptase (Invitrogen) and a forward primers specific for segment 4. Segment 4, containing the HA gene, was then amplified by PCR using segment 4-specific degenerated primer. After subcloning of the amplified cDNA into the pcDNA 3.1 vector (Invitrogen) and sequencing, the ORF was modified for baculovirus expression as described in Stevens et al. [4].
rHAs, stabilized by a his-tagged trimerization domain, were expressed into the supernatant of Baculovirus infected SF9 cells at 28 °C and harvested 4 d post infection. Soluble HA was recovered from the cell supernatant by metal affinity chromatography using NiNTA beads (GE Healthcare).[5] Fractions containing HAs were pooled and concentrated with Centrifugal Filter Unit (Ultra-15 Ultracel-10 membrane, Amicon) proteolytically processed into HA1 and HA2 with 10 U/µg HA of TPCK-treated trypsin (Sigma Aldrich) at RT for 1 h. Trypsin was removed by size exclusion chromatography on Superdex S200 slurry (GE Healthcare) and fractions containing HA trimers were collected and concentrated.

ELISA

96-well Corning® half-area polystyrene high binding microplates (Costar) were coated with 25 µl of 1 µg/mL HA in PBS at 4 ^{0}C for 64 h. Coated plates were then blocked with 3% BSA in PBS for at least 1h at RT. Sera were titrated in 1% BSA/PBS, and allowed to bind for 1 h. After washing with 0.01% Tween/TBS, bound serum IgG was detected with HRPO-coupled a goat anti-human fab-HRP antibodies (Jackson Immuno). The assay was developed for 12 min using 50 µL TMB (2 mg/mL) / H_2O_2 (5 µL/mL) in 0.1 M $NaH_2PO_4 * H_2O$ as a chromogenic substrate before the reaction was stopped by the addition of 50 µL 2 N H_2SO_4. After determining absorption at 450 nm, the serum dilutions giving half maximal absorbance was determined by non-linear regression of the measured OD values to the logarithmized serum concentration using the Hill-Curve as equation template. If no fit could be obtained, i.e. when an individual sample did provide sufficient data points for an accurate fit, top and bottom values were constrained to the corresponding average values determined over the whole assay fitting data to sigmoid dose response curves (variable slope) using GraphPad Prism 5 and 6 and Excel 2007 (Microsoft).

Neutralization assay

Strains used for neutralization assays were A/Puerto Rico/8/34(H1N1) (H1/34), A/Brisbane/59/07(H1N1) (H1/07), A/Singapore/1/57(H2N2) (H2/57), A/Brisbane/10/2007(H3N2) (H3/07), A/Hong Kong/68(H3N2) (H3/68), A/Duck/Czechoslovakia/56(H4N6) (H4/56), rg-A/Chicken/Vietnam/C58/2004(H5N3) [R] (H5/04) and A/FPV/Bratislava/79(H7N7) (H7/79).

Sera were titrated and combined with virus corresponding to an MOI of 2 to 6 in 20 mM Hepes supplemented DMEM containing 0.2 % BSA (DMEM/BSA) with the help of a semi-automated pipetting system (BioTek Precision). The virus-sera mix was incubated at 37 °C for 1 h and transferred to the cells, where non-neutralized virus was allowed to infect cells for 1 h at 37 °C. The virus-serum mix was then removed, plates washed with PBS and DMEM/BSA added. Viral protein synthesis was allowed to proceed for 6-7 h before cells were fixed and permeabilized with 100% methanol. Cells were then stained with FITC-labeled anti-NP antibody (3 μg/ml), H16-L10-4R5 (ATCC No. HB-65™), in PBS containing 1% BSA at 4 °C over night, followed by nuclear staining using Hoechst dye (Molecular probes). Fluorescence or both FITC and Hoechst were measured by a Perkin Elmer plate reader at 16 different locations in each well to account for variation in the local cell densities. The average from the individual measuring points was used to calculate the half maximal inhibitory concentration (IC_{50}) were determined using the same approach as described for EC_{50}.
For H5 and H7 low heterosubtypic neutralizing activity was found in sera that prevented computation of the logIC_{50}. Instead, the inhibition percentage at the first dilution (1 in 90) was taken as comparative value.

Supplementary References

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